

# Regulation of 1-Cys Peroxiredoxin Expression in the Process of Stromal Wound Healing after Photorefractive Keratectomy

Hungwon Tchah, Myoung Joon Kim, Tae-im Kim, Hyun-jeung Choi, Jae Yong Kim, Mi Jung Kim, and Jhang Ho Pak

**PURPOSE.** To investigate 1-cys peroxiredoxin (1-cysPrx) expression during the corneal wound-healing process after PRK and the effect of growth factors on 1-cysPrx expression in cultured bovine keratocytes (BKs).

**METHODS.** Rat corneas were excised at 4 hours, 12 hours, 1 day, 3 days, and 7 days after PRK. Expression of 1-cysPrx in the corneas was examined by immunohistochemical, Northern blot, and immunoblot analyses. Keratocytes were isolated from bovine corneas and subcultured to study the effects of TGF- $\beta$ 1, keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and H<sub>2</sub>O<sub>2</sub> on 1-cysPrx expression at different concentrations and time intervals. Generation and proliferation of intracellular reactive oxygen species (ROS) in cultured BKs stimulated by these growth factors were measured by the DCF (2',7'-dichlorofluorescein) assay, the CCK-8 assay, and immunoblot analysis with a polyclonal proliferating cell nuclear antigen (PCNA) antibody, respectively.

**RESULTS.** Intense staining of 1-cysPrx was observed in the epithelia and the anterior stromas of wounded corneas 4 hours after PRK and had extended to the entire stroma by day 3. By day 7, the expression almost returned to nonsurgical control level in epithelia, although notable expression was still detectable in the stroma. Concomitant augmentation of 1-cysPrx mRNA and protein was seen in the corneas at 12 hours to 7 days. Growth factor treatment in cultured BKs resulted in 1-cysPrx induction in a dose- and time-dependent manner. Growth factor-stimulated cells showed strong DCF fluorescence and increased proliferation during a 24-hour incubation, during which an upregulation of 1-cysPrx occurred.

**CONCLUSIONS.** These observations provide new information for the regulation of 1-cysPrx expression during the corneal wound-healing process. (*Invest Ophthalmol Vis Sci.* 2005;46:2396-2403) DOI:10.1167/iovs.05-0107

The cornea is a transparent tissue that consists of three cellular layers: epithelial, stromal, and endothelial. It functions as the primary optical component and as an initial barrier

to tears and intraocular environments. To correct myopia and astigmatism, the refractive power of the eye can be changed by removing corneal tissue with a 193-nm excimer laser in a technique known as photorefractive keratectomy (PRK).<sup>1,2</sup> Once the corneal epithelium is damaged by trauma or refractive surgery, a series of wound-healing processes begins, with cytokine-mediated interactions between epithelial cells and keratocytes. The initial stage of wound healing in the cornea involves apoptosis of the keratocytes, which occurs in response to the release of interleukin (IL)-1, Fas/Fas ligand, or tumor necrosis factor (TNF)- $\alpha$  by the injured epithelium.<sup>3-5</sup> After apoptosis, the remaining keratocytes begin to proliferate and migrate into the area of the healing wound, which may lead to myofibroblast differentiation.<sup>6,7</sup> Reactivation and repopulation of keratocytes may affect surgical outcomes, including corneal opacity and myopic regression. To prevent postoperative complications, researchers have attempted to regulate the growth factors and their receptors that are responsible for wound healing after the injury. Also, the development of drugs designed to control apoptosis and proliferation is in progress. However, little is known about the genes that participate in the wound-healing process, including apoptosis, proliferation, and differentiation.

The peroxiredoxins (Prx), a relatively new family of antioxidant enzymes, catalyze the reduction of a broad spectrum of peroxides through conserved cysteine residues.<sup>8-10</sup> Of the six mammalian Prx enzymes, five (2-cysPrx and Prx types I through V) contain two reactive cysteines and use thioredoxin as a reductant. By contrast, 1-cysPrx (Prx VI) is the only member that has a single conserved cysteine in the peroxide reduction active site.<sup>11,12</sup> 1-cysPrx has been isolated from bovine ciliary body,<sup>13,14</sup> rat olfactory epithelium,<sup>15</sup> and rat and bovine lung.<sup>16,17</sup> Overexpression of 1-cysPrx can protect NCI-H441 cells against phospholipid peroxidation-mediated membrane damage,<sup>18</sup> whereas the accumulation of phospholipid hydroperoxide and apoptosis of lung epithelial cells occur when endogenous expression is blocked by treatment with an antisense oligonucleotide to 1-cysPrx.<sup>19</sup>

The presence of 1-cysPrx is distributed throughout all ocular tissues, including the cornea.<sup>20</sup> However, its physiological function in the eye remains uncertain. In the present study, we investigated the levels of 1-cysPrx expression in rat corneas during the wound-healing process that follows PRK. We also examined the effect of growth factors on the expression of this protein in cultured bovine keratocytes (BKs). Growth factors were chosen for study because they are expressed in various corneal cells and play key roles in corneal wound healing. The results provide new information for the regulation of 1-cysPrx expression during the corneal repair process, thereby contributing to our understanding of Prx proteins as multifunctional enzymes which are not limited to their peroxidase activity.

## MATERIALS AND METHODS

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Hanks' balanced salt solution (HBSS), phosphate-buffered

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saline (PBS), trypsin-EDTA, and penicillin-streptomycin were purchased from Life Technologies (Gaithersburg, MD). TGF- $\beta$ 1, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and keratinocyte growth factor (KGF) were from R&D Systems (Minneapolis, MN). Mouse monoclonal anti- $\beta$ -actin and H<sub>2</sub>O<sub>2</sub> were obtained from Sigma-Aldrich (St. Louis, MO). Bovine polyclonal proliferating cell nuclear antigen (PCNA) antibody was obtained from Chemicon (Temecula, CA). Polyclonal 1-cysPrx antibody and bovine 1-cysPrx cDNA clone were generously provided by Aron B. Fisher (Institute for Environmental Medicine at the University of Pennsylvania Medical Center, Philadelphia, PA). All chemicals (biotechnology grade) were purchased from Amresco, Inc. (Solon, OH).

### Photorefractive Keratectomy

Animal care and experimental procedures were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Forty adult Sprague-Dawley male rats (~250 g) were anesthetized by intramuscular injection of ketamine (20 mg/kg) and xylazine (10 mg/kg). After a drop of proparacaine, transepithelial PRK (4-mm optical zone, -10 D) was performed on the right eye with a flying spot excimer laser (Technolas 217z; Bausch & Lomb Surgical, Munich, Germany). After excimer laser treatment, ofloxacin ointment was applied. At 4 hours, 12 hours, 1 day, 3 days, and 7 days after PRK, eight rats were randomly selected and euthanized. Eyes were enucleated and rinsed with cold PBS twice. The corneas were subsequently excised under an operating microscope, immediately frozen at -80°C until RNA and protein extraction, or fixed overnight in 4% paraformaldehyde in PBS for immunohistochemical analysis. At each time point, the left eyes were used as the control.

### Immunohistochemistry

Paraffin-embedded sections were prepared from fixed tissues and mounted on microscopic slides. Slides were deparaffinized and rehydrated with xylene and a graded series of ethanol. Sections were stained by sequential incubation with 1-cysPrx antibody (1:600 dilution in PBS containing 0.3% Triton X-100 and 3% bovine serum albumin) followed by incubation in Texas red-conjugated goat anti-rabbit IgG (diluted 1:1000 in the same buffer). After extensive washes in PBS with 0.3% Triton X-100 and in PBS, the sections were air dried, and coverslips were sealed with aqueous mounting medium. As a control for nonspecific binding, nonimmune serum (diluted 1:600) was substituted for the primary antibody. All sections were photographed with an inverted microscope (DMIRE2; Leica, Heidelberg, Germany) equipped with a confocal laser scanning system (TSC-SP2; Leica). For quantitation, we collected the fluorescent images with a single rapid scan with identical parameters (such as brightness and contrast) for each sample.

### Bovine Keratocyte Culture

Fresh bovine eyeballs from a local abattoir were soaked in 5% povidone iodine solution for 10 minutes and washed three times with PBS. The epithelium and endothelium were removed manually from the cornea, and the stroma was minced into pieces of ~5 mm<sup>3</sup>. Stromal explants were cultured in DMEM supplemented with 10% FBS and an antibiotic mixture at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Keratocytes were allowed to migrate from the explants to the surface of culture plates. The cells reached confluence within ~20 days. After the organs were discarded, the cells were detached and subcultured in 35-mm dishes. Cells from cultures between the third and sixth passage were used in the following experiments.

For the experiments involving growth factor or H<sub>2</sub>O<sub>2</sub> exposure, 5 × 10<sup>5</sup> keratocytes were seeded on a 35-mm culture plate and grown for 24 hours in standard growth conditions. Cells were gradually deprived of serum by incubation in 1% FBS overnight, followed by incubation in serum-free medium for 3 hours before use. These serum-starved cells were treated with various concentrations of TGF- $\beta$ 1 (2–20 ng/mL), KGF (2–20 ng/mL), HGF (2–20 ng/mL), PDGF (5–100 ng/mL),

or H<sub>2</sub>O<sub>2</sub> (25–75  $\mu$ M) and incubated for 12 hours. Cells were also exposed to fixed concentrations of growth factors and incubated for the indicated times. The concentrations of growth factors and the time points used in this study fell well within the range used in other studies examining growth factor-induced reactive oxygen species (ROS) generation and proliferation in multiple cell types.<sup>21–24</sup>

### Immunoblot Analysis

Frozen corneas (two for each time point) or growth factor-treated keratocytes were disrupted with lysis buffer (25 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol [DTT], 10% glycerol, 1% Triton X-100, and complete protease inhibitor cocktail). The extracts were homogenized by sonication and centrifuged at 10,000g for 20 minutes at 4°C. Protein concentrations were determined with a protein assay (DC Protein Assay; Bio-Rad, Hercules, CA). Supernatants containing 20  $\mu$ g of proteins were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The blot was probed with polyclonal 1-cysPrx antibody (1:3000 dilution) and horseradish-peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution; Jackson ImmunoResearch Laboratory, West Grove, PA), followed by detection with enhanced chemiluminescence (ECL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and quantitation by densitometric scanning of the x-ray film with a fluorescence imager (FluorS Multi-Imager; Bio-Rad). The membrane was then washed with stripping solution (62.5 mM Tris-HCl [pH 6.8], 100 mM  $\beta$ -mercaptoethanol, and 2% SDS) at 55°C and reprobed with monoclonal  $\beta$ -actin antibody (1:10,000 dilution) to normalize for protein loading.

### Northern Blot Analysis

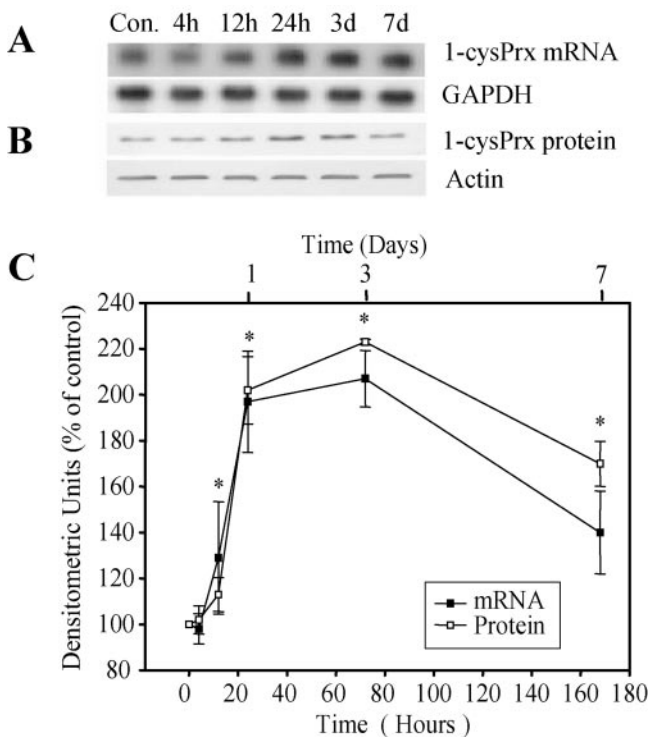
Total RNA was extracted from the corneas, or from growth factor- or H<sub>2</sub>O<sub>2</sub>-treated keratocytes using a kit (RNeasy mini kit; Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (5  $\mu$ g) was separated by electrophoresis on a 1% agarose gel containing formaldehyde, then transferred onto a nylon membrane (Schleicher & Schuell, Keene, NH) by capillary action and hybridized to a <sup>32</sup>P-labeled bovine 1-cysPrx cDNA probe generated by random priming (Amersham Biosciences). After a high-stringency wash, the membrane was exposed to x-ray film with an intensifying screen at -70°C for 14 hours and quantitated using the same method. To normalize for loading, the membrane was stripped of the 1-cysPrx probe, re-hybridized with a <sup>32</sup>P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe, and re-exposed to x-ray film.

### DCFH-DA Assay for Intracellular Reactive Oxygen Species

BKs were seeded at a density of 1 × 10<sup>4</sup> cells per well in a 96-well culture plate and were gradually deprived of serum in phenol-red-free DMEM. Cells were then washed once with HBSS and loaded with 50  $\mu$ M DCFH-DA (2',7'-dichlorofluorescein diacetate; Molecular Probes, Inc., Eugene, OR) in this buffer for 15 minutes. Cells were subsequently washed twice with HBSS and stimulated with growth factors (10 ng/mL of TGF- $\beta$ 1, KGF, HGF, or PDGF) or treated with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> as a positive control for 30 minutes at 37°C. Intracellular fluorescence was measured with a microplate fluorometer (Molecular Devices Corp., Sunnyvale, CA) with an excitation of 485 nm and an emission of 538 nm.

### Cell Proliferation Assay

The effect of growth factors on the proliferation of BKs was examined by a colorimetric cell-counting kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's instructions. Cells were cultured in a 96-well plate and stimulated as described earlier for 12 and 24 hours. Cells were then treated with 10  $\mu$ L CCK-8 solution/well and incubated for 2 hours at 37°C. The amount of formazan dye generated by cellular dehydrogenase activity was mea-



**FIGURE 1.** Expression of 1-cysPrx in rat corneas after PRK as detected by Northern (A) and immunoblot (B) analyses. Corneas that were obtained from rats between 4 hours and 7 days after PRK were evaluated. (A) Changes in 1-cysPrx mRNA levels, as analyzed by Northern blot. (B) Changes in protein levels, as analyzed by immunoblot. (C) Individual data were quantified as densitometry units and normalized with GAPDH (for mRNA) or actin (for protein). Data expressed as percentages (relative to the control) and are the mean  $\pm$  SE of results in three separate rat corneas at each time point. \* $P < 0.05$  compared with the nonsurgical control.

sured by absorbance at 450 nm with a microplate fluorometer. Absorbances were converted to percentages for comparison with the untreated control. The expression level of PCNA in the stimulated cells was also evaluated by immunoblot analysis with the appropriate antibody.

### Data Analysis

The data are expressed as the mean  $\pm$  SE. Statistical analyses were performed with Student's *t*-test. The level of significance was considered to be  $P < 0.05$ .

## RESULTS

### 1-cysPrx Expression in Rat Corneas after PRK

Levels of both 1-cysPrx mRNA and protein increased in rat corneas within 12 hours of PRK (Fig. 1). Specifically, expression reached its highest level ( $\sim 200\%$ ) between 1 and 3 days after PRK. An apparent decrease in expression occurred on day 7, but was still high compared with the control corneas.

### Immunohistochemical Detection of 1-cysPrx in Rat Corneas after PRK

As measured by immunofluorescence, expression of 1-cysPrx was detectable in the epithelium, but was low in the stroma of the nonsurgical control corneas (Fig. 2A). As early as 4 hours after the injury, intense 1-cysPrx signal was observed in the epithelium and the anterior portion of the stromal wound area

(Fig. 2B). At 12 hours, immunoreactivity was extended into the central stromal region (Fig. 2C). The number of 1-cysPrx-positive keratocytes significantly increased throughout the stroma on days 1 and 3 (Figs. 2D, 2E). It is also noteworthy that strong fluorescence was present in the inner layer of the epithelium, corresponding to proliferating cells. By day 7, the expression of 1-cysPrx had almost returned to control levels in the epithelium, although 1-cysPrx-positive keratocytes were still present in the stroma (Fig. 2F). 1-cysPrx-positive cells were not detected in the negative control (Fig. 2G).

### Effect of TGF- $\beta$ 1 on 1-cysPrx Expression in BKs

We examined the effect of TGF- $\beta$ 1 on 1-cysPrx expression in cultured BKs. Serum depletion had no significant effect on 1-cysPrx expression during the experimental incubation periods (data not shown). Keratocytes were exposed to TGF- $\beta$ 1 at concentrations of 2 to 20 ng/mL for 12 hours. There was a concentration-dependent increase in 1-cysPrx expression within 8 ng/mL (Fig. 3A). Treatment with 8 ng/mL TGF- $\beta$ 1 resulted in a time-dependent induction of the mRNA and protein, with a significant increase noted at 6 hours and a further increase up to 24 hours (Fig. 3B). These increases were statistically significant compared with the untreated control keratocytes ( $P < 0.05$ ).

### Effect of KGF on 1-cysPrx Expression in BKs

Because 1-cysPrx was identified as a highly inducible gene by KGF in human skin cells,<sup>25</sup> we also investigated whether 1-cysPrx was upregulated by KGF in keratocytes. The cells treated with 2 to 20 ng/mL KGF for 12 hours showed that induction was achieved in a dose-dependent manner, with the maximum level of 1-cysPrx expression at 20 ng/mL (Fig. 4A). With 8 ng/mL KGF, mRNA levels increased by  $\sim 100\%$  at 9 hours and  $\sim 250\%$  at 24 hours relative to the untreated control. Meanwhile, the protein level increased by  $\sim 200\%$  at 9 hours and remained constant between 9 and 24 hours (Fig. 4B). These changes were statistically significant ( $P < 0.05$ ).

### Effect of HGF on 1-cysPrx Expression in BKs

To determine the effect of HGF on 1-cysPrx expression, keratocytes were exposed to 2 to 20 ng/mL of HGF for 12 hours. At 5 ng/mL, HGF increased 1-cysPrx mRNA and protein by  $\sim 50\%$  and  $\sim 100\%$ , respectively (Fig. 5A). There were no further increases in expression with higher concentrations. A time course study with 5 ng/mL showed a  $\sim 25\%$  increase in expression at 3 hours, followed by gradual increases (up to  $\sim 100\%$ ) during longer incubations (Fig. 5B).

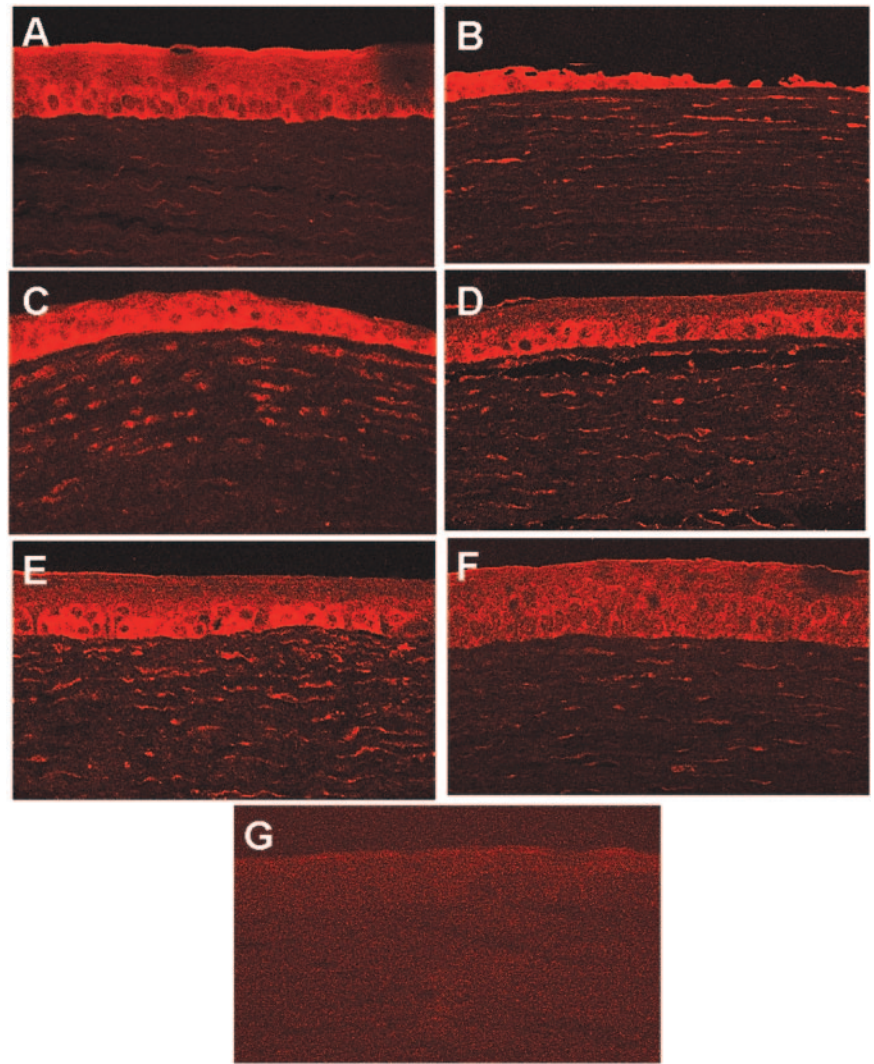
### Effect of PDGF on 1-cysPrx Expression in BKs

To evaluate the effect of PDGF on the expression of 1-cysPrx, cultured BKs were treated with PDGF at concentrations of 5 to 100 ng/mL for 12 hours. The expression of both mRNA and protein increased in response to PDGF doses as low as 5 ng/mL and also increased in a dose-dependent manner (Fig. 6A). Treatment with 10 ng/mL resulted in a time-dependent induction of expression, with a significant increase noted at 6 hours and an even further increase noted at 24 hours (Fig. 6B). There was no increase in mRNA beyond 15 hours, but a gradual increase in protein was observed.

### Intracellular ROS Generation Stimulated by Growth Factors in BKs

The generation of ROS has been detected in nonphagocytic cells stimulated by various cytokines.<sup>26</sup> Therefore, we assessed the production of intracellular generation of ROS in BKs stimulated by each growth factor, by using the oxidation-sensitive





**FIGURE 2.** 1-cysPrx immunofluorescence in rat corneas after PRK. The time points were 4 hours (B), 12 hours (C), day 1 (D), day 3 (E), and day 7 (F) after PRK or before PRK (A). Corneas that were exposed to rabbit nonimmune serum instead of primary antibody were used as the negative control (G). Fluorescent images were taken by a confocal microscope. Magnification,  $\times 400$ .

fluorescent probe DCFH-DA. Levels of DCF fluorescence increased by  $\sim 40\%$  in response to growth factors, comparable with that of  $50 \mu\text{M}$   $\text{H}_2\text{O}_2$  exposure (Fig. 7A). To examine the ROS as inducers for 1-cysPrx expression, BKs were exposed to  $\text{H}_2\text{O}_2$  at concentrations of 25 to  $75 \mu\text{M}$  for 12 hours, which had no effect on the release of lactate dehydrogenase (data not shown). There was a concentration-dependent increase in 1-cysPrx mRNA expression up to  $\sim 160\%$  (Fig. 7B).

### 1-cysPrx Induction in Proliferating BKs Stimulated by Growth Factors

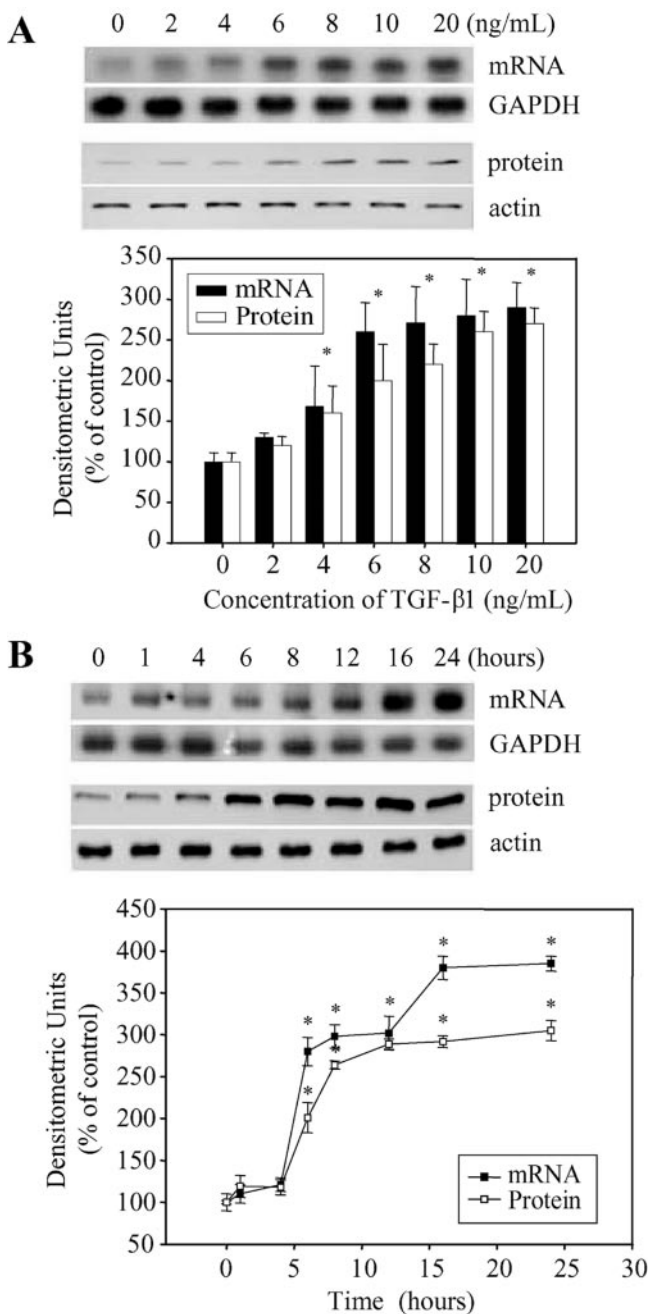
To observe the augmented presence of 1-cysPrx in actively proliferating cells, we first examined the effect of growth factors on cellular proliferation by using a CCK-8 assay. As shown in Figure 8A, there were no changes in the proliferative rate of untreated control cells during a 12 hour or 24 hour incubation due to serum starvation, whereas significant ( $P < 0.05$ ) increases were present in TGF- $\beta 1$ -, KGF-, and PDGF-stimulated cells. The effect HGF was significant at 24 hours, but not at 12 hours. Proliferation was confirmed by an immunoblot for PCNA, an excellent marker for mitogenic activation. Consistent with results of the CCK-8 assay, stimulated cells produced markedly higher levels of immunoreactive PCNA at 24 hours (Fig. 8B). PDGF induced PCNA the most ( $\sim 400\%$ ), followed by TGF- $\beta 1$  ( $\sim 200\%$ ), KGF ( $\sim 60\%$ ), and HGF ( $\sim 40\%$ ).

The response of 1-cysPrx toward each growth factor was similar to that of PCNA, which were  $\sim 300\%$  by PDGF,  $\sim 200\%$  by TGF- $\beta 1$ ,  $\sim 150\%$  by KGF, and  $\sim 80\%$  by HGF, respectively.

### DISCUSSION

The present study addressed the hypothesis that 1-cysPrx plays a major role in cellular response to injury by evaluating the levels of 1-cysPrx expression in an in vivo system. Because changes in immunoreactive 1-cysPrx in the stroma after PRK were dramatic (compared with those in the epithelium) and were maintained through the entire repair period, our subsequent studies to determine the effect of growth factors on 1-cysPrx expression were performed using cultured BKs.

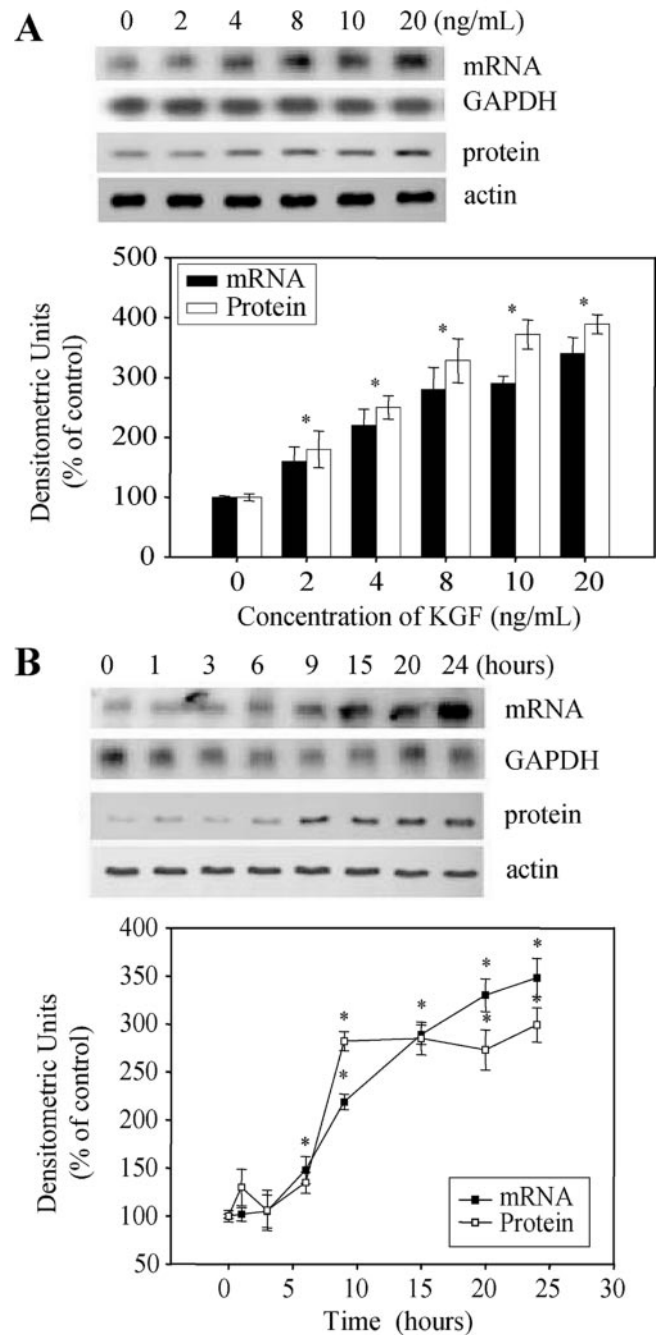
Various growth factors have been detected in the cornea, where they play unique and important roles in homeostasis and wound-healing regulation.<sup>27,28</sup> For example, expression levels of epidermal growth factor (EGF), HGF, KGF, and their receptor mRNAs were low in unwounded mouse corneas. After wounding, however, the growth factor mRNA expression was markedly upregulated in the keratocytes, even after closure of the epithelial defect.<sup>29</sup> In stroma and epithelium, the profile of the increase in 1-cysPrx protein after PRK essentially coincided with those of the growth factors, which suggests that de novo synthesis of 1-cysPrx may play an important role



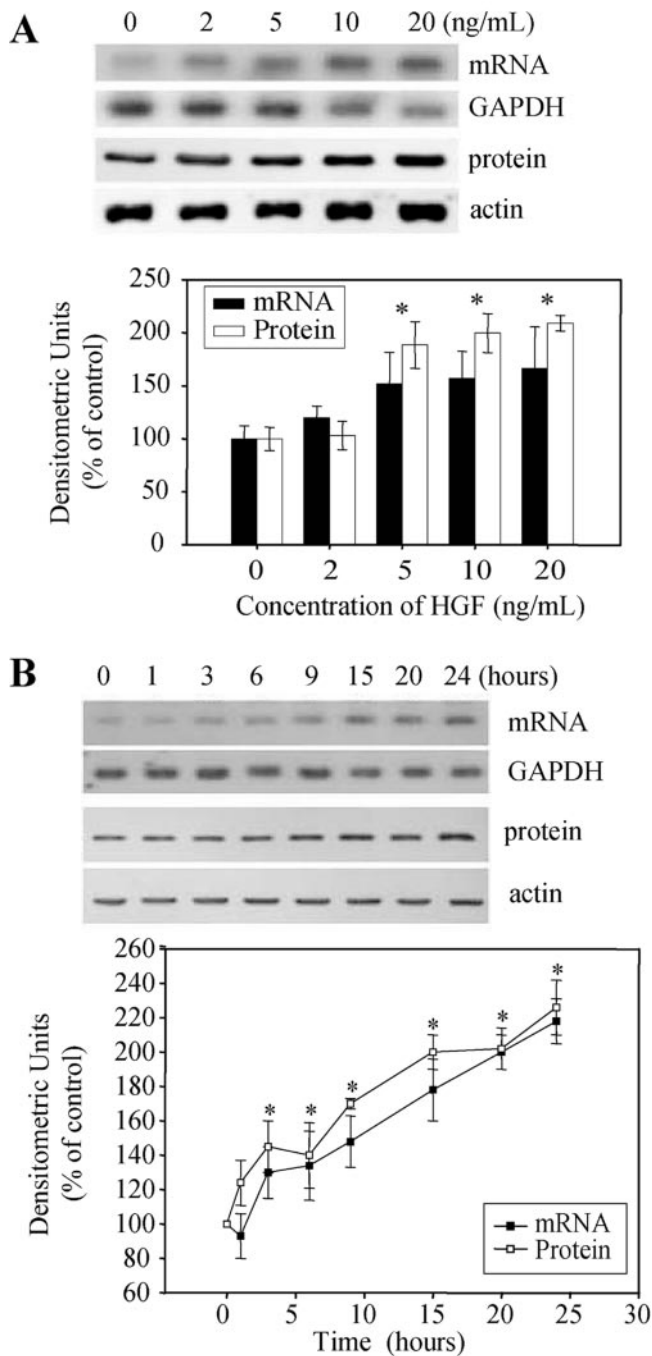
**FIGURE 3.** Effect of TGF- $\beta$ 1 on the expression of 1-cysPrx in BKs. Dose effect (A) and the time course studies for the effect of TGF- $\beta$ 1 on 1-cysPrx expression (B). (A) Cells were treated with TGF- $\beta$ 1 (2–20 ng/mL) for 12 hours and analyzed by Northern blot (for mRNA) and immunoblot (for protein), using GAPDH and actin as loading standards, respectively. (B) Cells were treated with 8 ng/mL TGF- $\beta$ 1 and harvested at times between 1 and 24 hours, followed by Northern and immunoblot analyses. Individual data were quantified as densitometry units and are presented as a percentage of the control (without treatment or time 0). Data are the mean  $\pm$  SE of results in three independent experiments. \* $P$  < 0.05 compared with the control.

in the mechanism of corneal wound healing. Previous studies have also shown an induction of 1-cysPrx after cutaneous injuries in mice, similar to that seen for KGF.<sup>30</sup> Expression was particularly abundant in the hyperproliferative keratinocytes at the wound's edge, suggesting an important role of the enzyme during the repair process. We monitored changes in 1-cysPrx expression in cultured BKs treated with TGF- $\beta$ 1, HGF, KGF, or

PDGF, resulting in an increase in expression in a dose- and time-dependent manner (Figs. 3, 4, 5, 6). Augmented expression was obvious in proliferating keratocytes during a 24-hour incubation with these growth factors (Fig. 8), suggesting the involvement of 1-cysPrx in cell proliferation. It is believed that



**FIGURE 4.** Effect of KGF on the expression of 1-cysPrx in BKs. Dose effect (A) and time course studies for the effect of KGF on 1-cysPrx expression (B). (A) Cells were treated with KGF at various concentrations for 12 hours and analyzed by Northern blot for mRNA and by immunoblot for protein using GAPDH and actin as loading standards, respectively. (B) Cells were treated with 8 ng/mL KGF and harvested at times between 1 and 24 hours, then subjected to Northern and immunoblot analyses. Individual data were quantified as densitometry units and are presented as a percentage of the control (without treatment or time 0). Data are the mean  $\pm$  SE of results in three independent experiments. \* $P$  < 0.05 compared with the control.

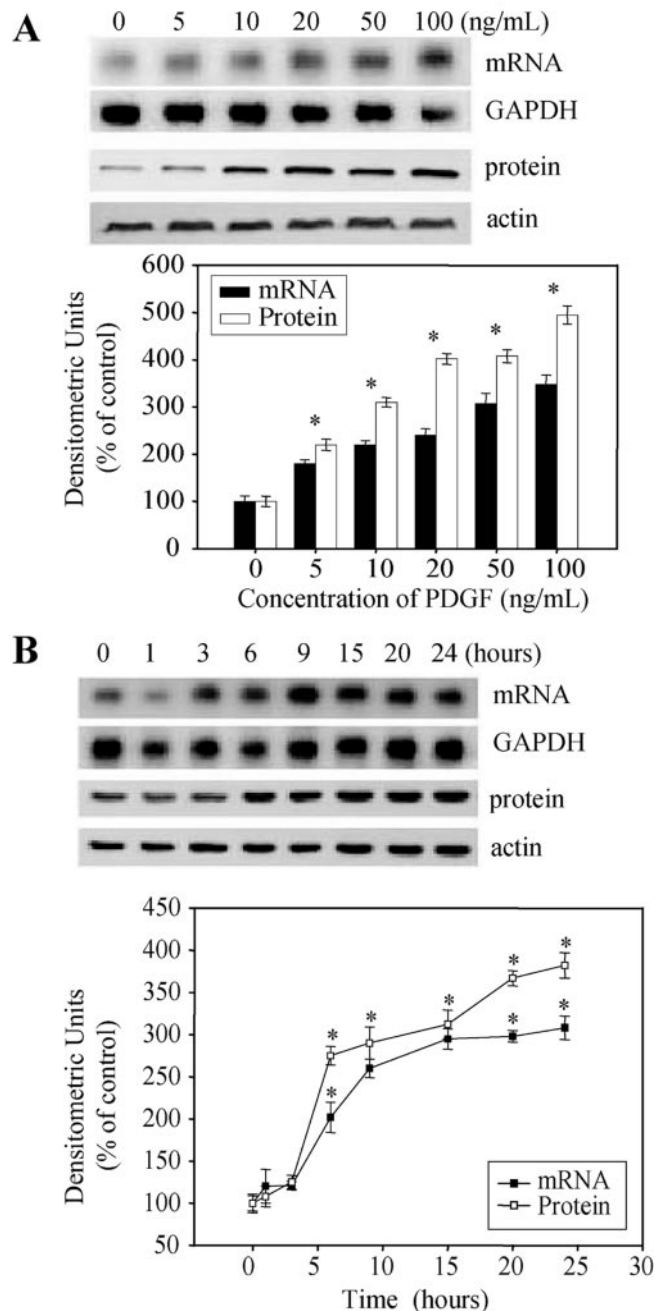


**FIGURE 5.** Effect of HGF on 1-cysPrx expression in BKs. (A) Dose effects of HGF. Cells were treated with HGF (2–20 ng/mL) for 12 hours and 1-cysPrx expression was analyzed by Northern blot and immunoblot. (B) Time course of HGF effects on 1-cysPrx expression. Cells were treated with 5 ng/mL HGF for 1 to 24 hours and harvested at various times for Northern and immunoblot analysis. Expression of 1-cysPrx mRNA and protein were normalized to GAPDH and actin and are presented as a percentage of the control (without treatment or time 0). Data are the mean  $\pm$  SE of results in three independent experiments. \* $P < 0.05$  compared with control.

KGF and HGF induce cellular proliferation and migration in the corneal tissues as paracrine mediators, whereas PDGF acts through both autocrine and paracrine mechanisms.<sup>8</sup> The most intriguing result is the mitogenic response of keratocytes to TGF- $\beta$ 1, which acts as an autocrine mediator. TGF- $\beta$  has been regarded as a multifunctional cytokine with the capacity to

promote or inhibit cell proliferation, depending on cell type. TGF- $\beta$ 1 significantly stimulates cell proliferation of cultured bovine and rabbit keratocytes.<sup>23,31</sup>

Many mammalian cells produce intracellular ROS in response to various cytokines, such as IL-1, TNF- $\alpha$ , IFN- $\gamma$ , TGF- $\beta$ , PDGF, HGF, KGF, EGF, and basic fibroblast growth factor.<sup>26</sup> The detection of intracellular ROS and the induction of

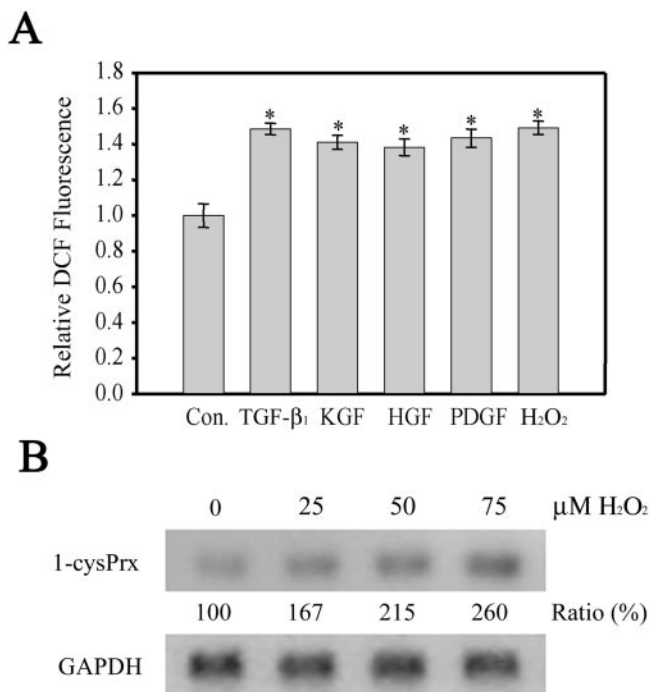


**FIGURE 6.** Effect of PDGF on 1-cysPrx expression in BKs. (A) Dose effects of PDGF. Cells were treated with PDGF (5–100 ng/mL) for 12 hours, and 1-cysPrx expression was analyzed by Northern blot and immunoblot analyses. (B) Time course of PDGF effects on 1-cysPrx expression. Cells were treated with 10 ng/mL PDGF for 1 to 24 hours and harvested at various times for Northern and immunoblot analysis. Expression of 1-cysPrx mRNA and protein were normalized to GAPDH and actin and are presented as a percentage of the control (without treatment or time 0). Data are the mean  $\pm$  SE of results in three independent experiments. \* $P < 0.05$  compared with the control.

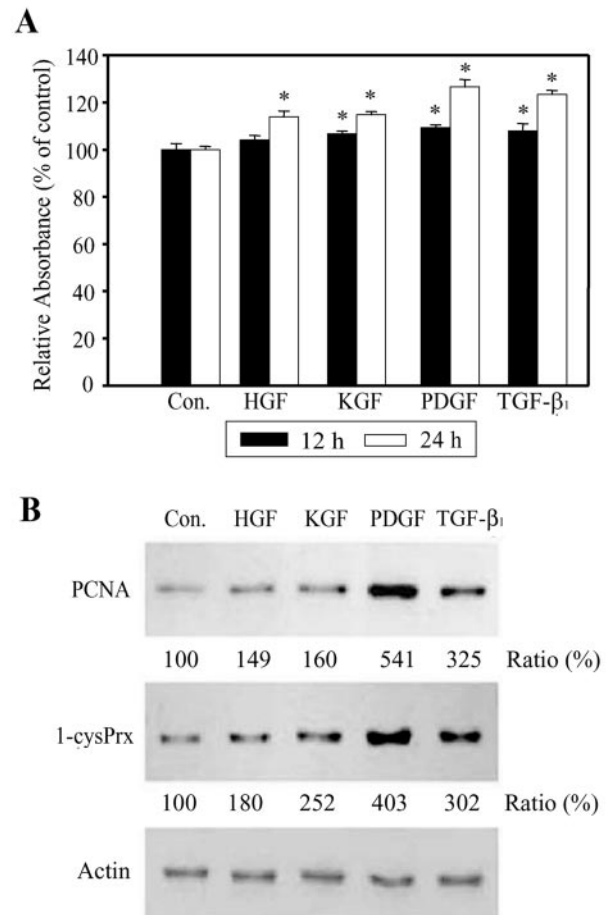


1-cysPrx in BKs during growth factor and  $H_2O_2$  exposure (Fig. 7) imply that the rapid removal of ROS and its production for signal cascades are necessary for the maintenance of redox homeostasis, for which 1-cysPrx may be responsible. Previously, transient overexpression of Prx I or II, members of the 2-cysPrx family, in A431 cells or NIH 3T3 cells resulted in reduced intracellular  $H_2O_2$  levels generated by EGF or PDGF. Moreover, the activation of NF- $\kappa$ B exposed to the extracellular addition of  $H_2O_2$  or TNF- $\alpha$  was attenuated by Prx II overexpression.<sup>21</sup>

Excimer laser ablation produces oxygen free radicals. This is probably due to UV radiation, polymorphonuclear cell infiltration, and thermal increase.<sup>32,33</sup> Previous investigators have studied the relative importance of antioxidant enzymes in the ability of corneal tissues to tolerate oxidative stress, focusing primarily on classic antioxidant enzymes, such as superoxide dismutase (SOD)<sup>34</sup> and glutathione peroxidase (GPx).<sup>35</sup> However, the activities of these enzymes decreased in rabbit corneas after different refractive corneal surgery,<sup>36,37</sup> speculating that another antioxidant enzyme may be necessary to compensate for the reduction in SOD and GPx levels. 1-cysPrx expression increased in corneal tissues as early as 4 hours after PRK and remained high until day 7 (Figs. 1, 2), suggesting that 1-cysPrx may be an important enzyme involved in migration, proliferation, and differentiation, which occur during corneal wound healing. It is also possible that 1-cysPrx may function as an antioxidant enzyme to reduce the accumulation of ROS caused by excimer laser or growth factors, thus preventing apoptotic cell death.



**FIGURE 7.** Measurement of growth factor-induced ROS generation by DCF fluorescence (A) and effect of  $H_2O_2$  treatment on 1-cysPrx mRNA expression (B) in BKs. (A) Cells were treated with each growth factor (10 ng/mL) or 50  $\mu$ M  $H_2O_2$  as a positive control for 30 minutes. Generation of ROS was determined by DCF fluorescence. Each data set represents relative values for untreated cells and means  $\pm$  SE for three independent experiments. \* $P$  < 0.05 compared with the control. (B) Representative Northern blot for 1-cysPrx expression in  $H_2O_2$ -exposed BKs. Cells were treated with  $H_2O_2$  at various concentrations for 12 hours and analyzed by Northern hybridization. Expression of 1-cysPrx mRNA was normalized to that for GAPDH, as indicated by the ratio below each lane.



**FIGURE 8.** Effect of growth factors on cellular proliferation and the induction of 1-cysPrx in BKs. (A) Proliferative response to each growth factor. Cells were stimulated with 10 ng/mL of various growth factors for 12 hours and 24 hours. Growth was then measured based on formazan dye production in a CCK-8 assay. Each data set represents a relative percentage of the control (without treatment). Data are the mean  $\pm$  SE of three independent experiments. \* $P$  < 0.05 compared with the control. (B) Representative immunoblot for PCNA and 1-cysPrx expression. Growth factor-stimulated cells were harvested after 24 hours and analyzed by immunoblot using PCNA and 1-cysPrx antibodies. Expression of PCNA and 1-cysPrx proteins were normalized to that for actin, as indicated by the ratio below each lane.

In human lens epithelial cells, 1-cysPrx was induced by lens epithelium-derived growth factor to protect cells from oxidative stress.<sup>38</sup> Other studies using the same cell line showed that overexpression of 1-cysPrx prevents hyperglycemia-mediated apoptosis, a result of osmotic and oxidative stress.<sup>39</sup> Recently, gene-targeted mice without 1-cysPrx have shown increased sensitivity to paraquat-induced oxidative injury, which was not replaced by other Prxs and antioxidant enzymes.<sup>40</sup> Taken along with the data in the present study, we postulate that 1-cysPrx may play divergent roles in the eye that are associated with various biological processes, such as oxidant detoxification, proliferation, and differentiation. These are functions that are not met by catalase, SOD, or GPx.

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